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(54) Title: THERAPY WITH 2-5A AND INTERFERON

(57) Abstract: New methods of treating malignancies in a subject are provided. Such methods comprise administering 2', 5' oligoadenylate (2-5A) or a biostable analog of 2-5A to the subject. In some embodiments, IFN is also administered to the subject. Examples of cancers which may be treated according to the present method include, but are not limited to, hairy cell leukemia, Kaposi's sarcoma, CML, B-cell and T-cell lymphomas, melanomas, myelomas, renal cell carcinoma, ovarian, breast, bronchogenic, bladder, and gastrointestinal carcinomas and acute leukemias, malignant glioma and fibrosarcoma. Methods of treating viral diseases in a subject are also provided. Such methods comprise administering interferon and 2', 5' oligoadenylate (2-5A) or a biostable analog of to a subject. Examples of viral diseases which may be treated according to this method include, but are not limited to, hepatitis C, hepatitis B, and viral infections caused by human papilloma virus or a picornavirus. A method of selectively inducing programmed cell death (apoptosis) in cancer cells either in vitro or in vivo is also provided. The method comprises administering 2-5A or a biostable analog thereof to the cancer cells.

THERAPY WITH 2-5A AND INTERFERON

Under 35 USC §119(e)(1), this application claims the benefit of prior U.S. provisional application 60/156,542, filed September 29, 1999.

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BACKGROUND

The interferons (IFNs) are a family of pleiotropic cytokines that are responsible for providing vertebrates with innate immunity against a wide-range of viruses and other microbial pathogens (Stark, et al., Annual Review of Biochemistry, 67, 227-264, 1998)). In addition, IFNs regulate cell proliferation, apoptosis and the immune system, properties that underlie their uses in treatment of cancer. IFNs alter patterns of gene expression in cells by binding to specific cell surface receptors and activating JAK/STAT pathways.

Type I IFN consists of the IFN -α,'-β, and -ω, all of which are structurally related and encoded in a gene cluster on the short arm of human chromosome 9 (Knight E. (1976), IFN: purification and initial characterization from human diploid cells, Porc. Natl. Acad. Sci. USA, 73:520-523). The Type I IFN genes are differentially regulated. All nucleated mammalian cells produce type I IFNs in response to viral infections. Type II IFN consists solely of IFN-γ, which is produced by T-cells and natural killer cells and has no structural similarity to type I IFNs (Young H., Hardy K. (1990), IFN-γ producer cells, activation stimuli and molecular genetic regulation, Pharmacol. Ther., 45:137-151). The human IFN γ gene is located on chromosome 12.

For the past 25 years IFNs, alone and in combination with other agents, have been used in the clinic against a wide range of human malignancies. For example, IFN-α2 as a single agent has been used to treat hairy cell leukemia, Kaposi's sarcoma, chronic myelogenous leukemia (CML) , B-cell and T-cell lymphomas, melanomas, myelomas, and renal cell carcinoma. In addition, IFN-α2 has been used in combination with doxorubicin for treating follicular

lymphoma. IFN-α2 has also been used in combination with 1-α-D arabinofuanosylctyosine to improve the cytogenic response in CML. IFNβ has been used to treat hepatocellular carcinoma. IFNs have also been used to treat various viral diseases including chronic active hepatitis and recurring papillomas.

While current IFN therapies have proved beneficial, their efficacy is somewhat limited, in part due to significant side-effects. Therefore, there is a need for additional anti-cancer and anti-viral therapies, particularly for therapies which enhance the efficacy of IFNs.

SUMMARY OF THE INVENTION

The present invention provides new methods of treating malignancies in a subject, particularly a human subject. Such methods comprise administering 2', 5' oligoadenylate (2-5A) or a biostable analog of 2-5A to the subject. As used herein the term "2-5A" refers to a small molecule having the formula px(A2'p)n5'A, where x =1 to 3 and n is greater than or equal to 2. In some embodiments, IFN is also administered to the subject. Interferon and 2-5A or the biostable analog thereof are administered concurrently or sequentially. Interferon and 2-5A or biostable analog thereof are administered separately in different pharmaceutical compositions or together in a single pharmaceutical composition. Interferon and 2-5A or the biostable analog thereof are administered in therapeutically effective amounts. Examples of cancers which may be treated according to the present method include, but are not limited to, hairy cell leukemia, Kaposi's sarcoma, CML, B-cell and T-cell lymphomas, melanomas, myelomas, renal cell carcinoma, ovarian, breast, bronchogenic, bladder, and gastrointestinal carcinomas, acute leukemias, malignant glioma and fibrosarcoma.

The present invention also relates to new methods of treating viral diseases in a subject, particularly a human subject. Such methods comprise administering interferon and 2', 5' oligoadenylate (2-5A) or a biostable analog of 2-5A to a subject. Interferon and 2-5A or the biostable analog thereof are administered concurrently or sequentially. Interferon and the 2-5A or the biostable analog thereof are administered in amounts sufficient to reduce the pathological effects of a given virus in the subject. Examples of viral diseases which may be treated according to this method include, but are not limited to, hepatitis C, hepatitis B, and viral infections caused by human papilloma virus or a picornavirus.

The present invention also relates to a method of selectively inducing programmed cell death (apoptosis) in cancer cells either in vitro or in vivo. The method comprises administering 2-5A or a biostable analog thereof to the cancer cells. In one embodiment, IFN is also administered to the cell.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic of the IFN-regulated system.

Figure 2 shows the structure of the trimeric species of 2-5A and the structure of the 2-5A analog, sp(A2'p)$_3$5'A2'p3'dC (2-5AdC).

Figure 3 shows the effect of IFN and 2-5A therapy on levels of apoptosis in ovarian carcinoma cells (HEY1B) and normal ovarian epithelial cells (NOE) as determined by TUNEL assays. HEY 1B and NOE cells were plated ($2x10^5$ cells/plate) in six-well tissue culture plates and incubated in the absence or presence of IFN (2,000 U/ml) overnight prior to transfection with either A2'p5'A or 2-5A (6 μM) in serum-free Opti-MEM for 3.5h. Samples were diluted in growth media 24h for TUNEL analysis.

Figure 4 shows the kinetics of apoptosis induced in HEY1B cells by the combination of IFN and 2-5A as determined by Annexin V binding assays. HEY 1B cells were plated ($2x10^5$ cells/plate) in six-well tissue culture plates and incubated in the absence or presence of IFN (2,000 U/ml) overnight prior to transfection with either A2'p5'A or 2-5A (6μM) in serum-free Opti-MEM for 3.5h and then placed in growth media. Cells were harvested at various times for Annexin V binding assays by FACS analysis.

Figure 5 shows the effect of 2-5A treatment on the death of human malignant glioma U373 cells, as determined by trypan blue exclusion assay. U373 cells were transfected with the trimeric species of 2-5A, ppp5'A2'p5'A2'p5'A, which was complexed with lipofectamine PLUS. Cell death was determined after incubation of the cells for 16 h by trypan blue exclusion assay and counting live and dead cells under a microscope with a hemocytometer.

Figure 6 shows the effect of 2-5A and IFN treatment on the viability of ovarian carcinoma cells (HEY1B) as determined using Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay. Data displayed is the mean and standard deviation of triplicate analysis for 24h (A), 48h (B), and 72h (C).

Figure 7 shows the effect of treatment with IFN and the 2-5A analog, 2-5AdC on the viability of ovarian carcinoma cells, as determined by trypan blue dye exclusion assay. HEY1B cells were incubated overnight the absence or presence of 2,000 units per ml of IFN-α2a. Subsequently, cells were transfected with 3 μM or 6 μM of 2-5AdC and incubated 24 h. Results are an average of three separate assays + standard deviation.

Figure 8 shows the effect of IFN and 2-5A therapy on the growth of human ovarian carcinoma in nude (athymic) mice. HEY 1B human ovarian carcinoma cells, 3 x 10^6/site, were injected into groups of 4 nude mice. When tumors reached volumes of 100 mm^3, they were directly injected daily with 50 μl of 5 μM 2-5A. On day 6, the 2-5 A treated tumors were injected with 1,000 U of IFN-α2a.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for treating malignancies and viral diseases in a subject, particularly a human subject. The methods comprise administering a small molecule known as 2-5A to the subject. Preferably, interferon is also administered to the subject. Such methods are particularly useful for treating ovarian carcinoma, malignant gliomas, and breast cancer.

In accordance with the present invention, it has been determined that a combination of IFN and 2-5A is highly effective in selectively inducing programmed cell death (apoptosis) of human ovarian cancer cells (HEY1B) without affecting the viability of normal ovarian epithelial cells. A combination of IFN and 2-5A was also highly effective in selectively inducing apoptosis of human malignant glioma U373 cells. The molecular mechanism of cell death induced by IFN and 2-5A was investigated. Interferon treatment of the HEY1B cells led to enhanced levels of cytochrome C. Introduction of 2-5A into HEY1B cells led to a rapid release of the cytochrome C from the mitochondria into the cytoplasm, and to an activation of caspases 3 and 9. The pan-caspase inhibitor BD-fmk and the caspase 3 inhibitor DEVD-fmk partially suppressed the IFN and 2-5A apoptotic response in human malignant glioma U373 cells.

FORMULATION

As used herein interferon refers to the type I interferons which include IFN-α, with all of its subtypes, IFN-β, IFN-ω and IFN-τ, as well as the Type II interferon, IFN-γ. As used herein,

interferon also refers to IFN con, which is a consensus type I interferon produced by Amgen, and chemically-modified interferons such as pegylated interferon. Preferably, the interferon is a type I interferon, IFN con, or a chemically-modified version thereof.

2-5A is a small molecule having the formula px(A2'p)n5'A, where x =1 to 3 and n is greater than or equal to 2. 2-5A is a series of short, heat-stable oligoadenylates with unusual 2',5' phosphodiester linkages in contrast to the typical 3'-5' linkage that is found RNA and DNA chains (Kerr I. M., Brown R.E. (1978), pppA2'p5'A2'p5'A: an inhibitor of protein synthesis synthesized with an enzymes fraction from IFN-treated cells, Proc. Natl. Acad. Sci. USA, 75:256-260). Studies have shown that IFN induction of 2-5A synthetase via JAK-STAT signaling results in the formation of a mixture of 2-5A oligomers in the presence of dsRNA (from virus) and cellular ATP. It has also been shown that the trimeric and tetrameric forms of 2-5A bind to RNase L causing homodimerization and activation which results in the degradation of both cellular and viral RNA, effectively limiting viral replication (Figure 1) (Silverman R.H. (1997), In Ribonucleases: structure and functions, G. D'Alessio and J.F. Riordan, eds. New York, New York: Academic Press), pp. 515-551). In one embodiment, 2-5A is a mixture of oligomeric species. In other embodiments, 2-5A is a single species, e.g., the trimeric, tetrameric, or pentameric form of the oligoadenylate or an analog thereof. (See Figure 2)

Interferon and 2-5A from whatever source derived, including without limitation from recombinant and non-recombinant sources, may be administered separately in individual pharmaceutical compositions or together as a single pharmaceutical composition. Such composition or compositions may further comprise a pharmaceutically acceptable carrier. Such pharmaceutical composition may also contain diluents, fillers, salts, buffers, stabilizers, solubilizers, antioxidants and other additives well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the activity of the interferon or the 2-5A. The characteristics of the carrier will depend on the route of administration. The pharmaceutical composition may further contain other agents which either enhance the activity of interferon or 2-5A or complement its activity or use in treating the viral disease or malignancy. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with the interferon or 2-5A, or to minimize side effects.

The pharmaceutical compositions of the invention may be in the form of a liposome in which 2-5A is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art. In one embodiment, the 2-5A or analog is formulated with a cationic lipid carrier, such as lipofectamine or a cationic liposome. The cationic lipid carrier may further comprise a molecule which targets the resulting complex of carrier and 2-5A to a particular cell type, such as for example a tumor cell. Examples of such targeting molecules are folate, transferrin and antibodies or fragments thereof.

DOSAGE

Interferon and 2-5A are administered to a host subject in a therapeutically effective amount. As used herein, the term "therapeutically effective amount" means the total amount of interferon and that is sufficient to show a meaningful benefit, i.e., treatment, healing, or amelioration of the relevant malignancy or virally-induced disease, or an increase in rate of treatment, healing, or amelioration of such malignancy or disease.

The amount of interferon or 2-5A in the pharmaceutical composition will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the subject has undergone and the type of cancer or virus being targeted. Ultimately, the attending physician will decide the amount of 2-5A and interferon with which to treat each individual subject. Initially, the attending physician will administer low doses of 2-5A and interferon and observe the subject's response. Larger doses of 2-5A and interferon may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain from about 0.1 million to about 10 million units per dose of interferon and from about 1 mg to about 1 gm per dose of 2-5A or the analog thereof. Although a single dose of interferon and 2-5A may be sufficient to ameliorate the pathological effects of the virus or the malignancy, it is expected that multiple doses of interferon and the will be preferred.

MODES AND DURATION OF ADMINISTRATION

Administration of the pharmaceutical compositions comprising interferon, or 2-5A, or both may be carried out in a variety of conventional ways, such as cutaneous, subcutaneous, intramuscular, intraperitoneal, parenteral or intravenous injection. In one embodiment, the interferon pharmaceutical composition is administered first and then the 2-5A pharmaceutical composition is administered thereafter. In another embodiment, the 2-5A composition is administered first and the interferon containing composition is administered thereafter. When administered sequentially, the second composition is administered from several hours to about 3 day post treatment with the first composition. Alternatively, both the interferon and the 2-5A or 2-5A analog are administered simultaneously. Both agents may be administered simultaneously by continuous intravenous infusion. Alternatively the interferon may be administered by sub-cutaneous or intramusclar injection while 2-5A or 2-5A analog is administered at the same time by continuous infusion.

The duration of therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and response of each individual patient. Ultimately the attending physician will decide on the appropriate duration of therapy using the pharmaceutical composition of the present invention.

EXAMPLES

Exemplary methods are described below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present compositions and methods. All publications and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples are illustrative only and not intended to be limiting.

EXAMPLE 1 Treatment of Human Ovarian Cancer Cells with 2-5A and Interferon α2a.

2-5A was prepared using synthetase isolated from extracts of IFN-treated HeLa cells on an activating affinity matrix of poly(I):polyC-agrarose (AGPoly(I):poly(C)™-type 6; PharmaciaBiotech) (Silverman and Krause, 1985) grown in DMEM with 10% fetal bovine serum in ten 150 cm^2 plates at 37°C to a density of about 90% confluency and treated with 1,000 U/ml IFN α2a (Roferon, Roche) for 16h. Cells were washed in PBS and harvested by scraping. A

post-mitochondrial fraction was prepared by lysing the cells in 7 ml of buffer A (0.5% NP-40; 90 mM KCl; 1 mM Mg acetate, 10 mM Hepes, pH 7.6, 10μg/ml leupeptin and 2 mM 2-mercaptoethanol), vortexing briefly, and centrifuging at 10,000 g for 10 min. The supernatant containing the synthetase in crude extract was removed. Poly(I):poly(C)-agarose [1.2 ml suspension containing 6 mg of poly(I):poly(C)] was washed twice by centrifuging at 1,500 g for 5 min, discarding supernatant, and resuspending in 50 ml of buffer B (10 mM Hepes, pH 7.5; 50 mM KCl; 1.5 mM MgAC; 7 mM mercaptoethanol and 20% v/v glycerol). The poly (I):poly(C)Sepharose was resuspended in the post-mitochondrial fraction of Hela cells and incubated for 1 h at 4°C on a rotating shaker at a low speed. The complex of synthetase bound to the resin was centrifuged at 1,500 g for 5 min and the supernatant discarded. The pellet was washed three times in 15 ml of buffer B by centrifuging at 1,500 g for 5 min, discarding supernatant and resuspending. The final pellet was resuspended in Buffer B supplemented with 4 mM ATP adjusted to pH 7 (Sigma). The first round of synthesis was performed by incubation for 20h at 37°C with gentle, continuous shaking. The resin complex was separated from the crude 2-5A preparation by centrifuging and removing the supernatant containing the 2-5A. A second round of synthesis was performed by resuspending in fresh buffer B with 4 mM ATP and incubating an additional 37°C for 72h. The 2-5A was stored at -70°C.

2-5A was analyzed on a Beckman HPLC system using System Gold integration parameters. Crude extract was analyzed on a reverse phase HPLC column (C-18, 60A, 5μ Princeton Sphere) in a 50% methanol and ammonium phosphate (pH 7.2) mobile phase system ramping from zero to 25% methanol over 60 min. The crude preparation was compared to known standards of ATP (Sigma), dimeric (A2'p5'A, Sigma) and trimer ($p_3(A2'p)_2A$) forms for identification. Over 60% of the material consisted of the trimeric and tetramer forms of 2-5A while the remaining fraction consisted of ATP, dimeric, pentamer and hexamer.

Unless otherwise stated, "2-5A" as used in this example refers to this mixture of 2-5A oligomers.

Human HEY 1B cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin/penicillin. Normal ovarian epithelial cells (NOE) were cultured in a 1:1 (v/v) mixture of Medium 199 (with Earl's salts and L- glutamate without sodium bicarbonate) and MCDB-105 supplemented with 15% heat-inactivated fetal bovine serum and 10,000 units/ml penicillin/streptomycin, 10 ng/ml epidermal growth factor (Sigma). Cells were transfected with either 6 μM 2-5A or A2'p5'A

(Sigma), a 2-5A dimer which does not activate RNase L, using a Lipofectamine Plus (GIBCO/BRL) protocol in which 2-5A or the control dimer was incubated with Plus reagent for 15 min in serum-free Opti-MEM media prior to addition of lipofectamine. The combined mixture was incubated for an additional 15 min and diluted up to the appropriate volume in Opti-MEM. 1 ml of the transfection material was placed on individual wells of a six-well tissue culture plate seeded with 2 $x10^5$ cells, some of which had been pre-treated overnight with 2,000 U/ml IFN (Roferon α2a). The transfection material was incubated on cells at 37° C for 3.5h and an equal volume of DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin was added for the noted period of time.

Cells were transfected with 6 μM of a natural mixture of 2-5A and apoptosis was monitored by FACS TUNEL assays for DNA breakage. Cells were harvested at 24 h for TUNEL analysis by removing the transfection material and media to a 15-ml tube and rinsing the cells once with PBS. Cells were trypsinized and transferred to the same tube. The tissue culture wells were washed again and the wash was also added to the same tube. This material was centrifuged at 2,500 x g for 10 min, washed with PBS and centrifuged again. The cell pellet was resuspended in 1% paraformaldehyde prepared in PBS and incubated on ice for 1 min. The cells were centrifuged and washed prior to suspension in 70% ethanol. Samples were stored at -20°C. The APO-BRDU™ kit (Pharmingen, Becton Dickinson, San Jose, CA) was used to perform TUNEL analysis. The kit uses a two color staining method, one for labeling DNA breaks and another for labeling total cellular DNA. The enzyme, terminal deoxynucleotidyl transferase (TdT) catalyzes a template independent addition of brominated deoxyuridylate monophosphate (Br-dUMP) to the 3'-hydroxyl (-OH) ends of double- and single-stranded DNA. After Br-dUTP incorporation, sites are identified by an FITC-labeled anti-BrdU monoclonal antibody. Non-clumped cells are gated and separate boxes are drawn around cells that stain positive (upper box) and negative (lower box) with the FITC-BRDU mAb. Double-stained cells in the upper right quadrant determine the percent of apoptotic cells.

The results demonstrated that IFN treatment alone did not induce cell death. Similarly, an inactive analog, dimer (A2'p5'A) from Sigma Co., failed to induce apoptosis in the presence or absence of IFN. In contrast, by itself 2-5A induced 36.5% apoptosis and the combination of IFN followed by 2-5A induced 72.1% apoptosis. (Fig. 3)

To confirm that the cells were dying by apoptosis, Annexin V binding assays were performed (Fig. 4). In normal cells, phosphatidylserine (PS) is present in the cytoplasmic leaflet of the cell membrane. PS moves to the outer leaflet of the cell membrane as an early event in apoptosis. Annexin V binds to PS present on the cell surface and can be monitored using FITC-labeled annexin V with FACS analysis. Results were consistent with the TUNEL assays showing induction of annexin V binding by 16 hrs after treatment with 2-5A. The levels of annexin V positive cells was greatly increased by pretreating the HEY1B cells with IFN-α2 prior to transfections. There were 28.7% annexin V positive, apoptotic cells after 24 hr of 2-5A treatment whereas the combination of IFN and 2-5A caused 71.2% apoptosis. These results are in close agreement with the TUNEL assays. Therefore, IFN plus 2-5A induced HEY1B cells to undergo apoptosis, apparently in a highly selective manner.

To investigate why IFN pretreatment enhances apoptosis by 2-5A, we measured RNase L before and after IFN treatment. IFN induced RNase L levels in the HEY1B cells by about two-fold without enhancing rRNA cleavage. Therefore, alternative causes for the apoptotic enhancing effect of IFN were considered. Previously, in a screen for IFN regulated genes we discovered that cytochrome C mRNA levels were elevated after IFN treatment of HT1080 cells (Der et al., 1998). Cytochrome C is an important factor in the induction of apoptosis because after it is released from mitochondria it binds to Apaf-1 and activates procaspase 9 (Green and Reed, 1998; Reed 1997). We confirmed here that cytochrome C is an IFN induced protein using western blots. Cytochrome C levels increased after HEY1B cells were treated with 100 or 1,000 units per ml of IFN-α2a. Interestingly, the transfection of into HEY1B cells induced the rapid release of cytochrome C from the mitochondria into the cytoplasm. A sharp decrease in mitochondrial cytochrome C levels was observed within the first hour of 2-5A treatment. The molecular events by which leads to cytochrome C release from mitochondria is unknown. However, we have found RNase L in a mitochondrial fraction (P10).

EXAMPLE 2 Treatment of Human Ovarian Cancer Cells with 2-5A and IFNβ.

Human Hey 1 B cells were plated ($3x10^5$ cells/well) and treated daily with IFN β (0, 20, 100, or 1,000 U/ml) prior to transfection with 2-5A (0, 0.06, 0.12, 0.25, 0.5, or 1.0 μM) for a period of 24, 48, and 72 h. (Fig. 6). Cell viability was assessed using Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay. The CellTiter 96® AQueous Non-Radioactive Cell

Proliferation Assay (Promega) is a colorimetric method for determining the number of viable cells in proliferation or chemosensitivity assays. It is composed of solutions of a novel tetrazolium compound (3-(4,5dimethylthiazol-2-yl)-5-β-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS). MTS (Owen's reagent) is bioreduced by cells into a formazan that is soluble in tissue culture medium. The absorbance of the formazan at 490 nm can be measured directly from 96 well assay plates without additional processing. The conversion of MTS into the aqueous soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. At the indicated time after cell transfection, 50 μl of the MTS reagent (30% solution in PBS) was added to each well of the 96 well plate and incubated for 2h prior to monitoring the absorbance at 490 nn. Analyses was performed in triplicate and the background-corrected mean and standard deviation are shown.

To determine effects on cell viability, Hey1B cells were preincubated in the presence or absence of different concentrations of IFN-β for 16 h prior to daily transfections with different amounts of 2-5A for three days (Fig. 6). In the absence of IFN-β, 2-5A had a potent anti-cellular effect as determined with a colorimetric MTS tetrazolium assay (Promega). After 24, 48, and 72 h of treatment with 1 mM 2-5A there was 39, 60, and 90% reductions in cell viability, respectively. By the third day (72 h) of treatments, the IC50 for 2-5A was 0.5 mM. At 2-5A concentrations of >3 mM, no viable cells could be detected. IFN-β treatments also produced a dose-dependent anti-cellular effect. After four days, cell viability was reduced by 27, 54, and 85% with 20, 100 and 1000 units per ml of IFN-β, respectively. The combined treatments were particularly effective in decreasing cell viability. For instance, after 48 h of treatment with 2-5A (1 mM), IFN-b (1,000 units per ml), or the combination of 2-5A and IFN-β, cell viability was reduced by 60%, 78% and 96%, respectively. The enhanced anti-cellular effect of combining 2-5A and IFN-β treatments was further explored by monitoring apoptosis.

EXAMPLE 3 Treatment of Human Glioblastoma Cells with 2-5A in the Absence and Presence of IFN.

A mixture of 2-5A was prepared as described above in example 1 and used in

combination with IFNβ to treat human glioblastoma cells. The glioblastoma cell line, U373, was derived from a patient diagnosed with fatal and invasive brain cancer. These cells can induce tumors when injected in mice.

Prior to treatment, the 2-5A was assayed with purified, recombinant RNase L fusion protein. One of the most convenient sources of RNase L for functional analysis of 2-5A involves the *E. coli* expression and purification of a glutathione-*S*-transferase (GST) fusion protein. In a prior study, we described cloning a full-length coding sequence DNA for human RNase L downstream (3') of the coding sequence for GST in expression vector pGEX-4T-3 (Pharmacia Biotech). The cDNAs for RNase L in plasmid pGEX4-T-3 is transformed into *E. coli* strain DH5α and the bacteria grown at 30°C to A_{595} = 0.5 before being induced with 0.1 mM IPTG for 3 h. The harvested cell pellets are washed with PBS, resuspended in 5 volumes of PBS-C (PBS with 10% v/v of glycerol, 1 mM EDTA, 0.1 mM ATP, 5mM $MgCl_2$, 14 mM 2-mercaptoethanol, 1 μg per ml of leupeptin and 1 mM PMSF) supplemented with 1 μg per ml lysozyme, and incubated at room temperature for 20 min. The suspended cells are lysed by sonicating on ice for 20 sec four times, Triton-X 100 is added to a final concentration of 1% v/v and the cell lysates are incubated at room temperature for 20 min. The supernatants are collected after centrifugation at 16,700 g for 20 min at 4°C. Purification of fusion proteins is performed as described by the manufacturer of the glutathione-sepharose 4B (Pharmacia Biotech) with modifications. Briefly, glutathione-sepharose 4B (200 μl of a 50% slurry in PBS-C) is added to extract from 200 ml cultures of bacteria at room temperature for 20 min with shaking. After washing the protein-bead complexes three times with 10 ml of PBS-C, the fusion proteins are eluted with 20 mM of glutathione in 50 mM Tris-HCl, pH 8.0 containing 1 μg per ml leupeptin, with shaking at room temperature for 20 min. Expression and purity of the protein preparations are determined by SDS/PAGE and coomassie blue staining and by western blots with monoclonal antibody to RNase L.

As substrate, oligouridylic acid, U_{25}, (Midland Certified Reagent Co.) is labeled at its 3'-terminus with [5'-^{32}P]-pCp (3,000 Ci/mmole) (Du Pont NEN) with T4 RNA ligase (Gibco/BRL). The U_{25}-[^{32}P]pCp, 80-160 nM, is incubated with 100 nM (220 ng) of purified GST-RNase L in the presence and absence of different forms of 2-5A in a final volume of 20 μl at 30°C for 30 min. Reaction mixtures are heated to 100°C for 5 min in loading buffer and intact

RNA is separated from the RNA degradation products in 20% polyacrylamide/8% urea sequencing gels. The amount of intact U_{25}-[^{32}P]pCp remaining after the incubations can be determined from autoradiograms of the gels with a Sierra Scientific high resolution CCD camera (Sunnyvale, CA) and the computer program, NIH Image 1.6 or by phosphorimager (Molecular Dynamics).

In a representative assay, there was no RNA cleavage in absence of , or with $p_3A2'p5'A$ at concentrations as high as 100 nM. In contrast, 0.1 nM of $p_3A2'p5'A2'p5'A$ caused nearly complete cleavage of the U25 substrate. The mixture, 2nd round, was less active (by a factor of <10) than the purified trimer, $p_3A2'p5'A2'p5'A$. However, the mixture more closely mimics what is present in cells exposed to IFN and infected with virus.

Cell viability following transfections with trimeric or inactive dimeric control was assessed using trypan blue dye exclusion assays. U373 cells were cultured in six well plates ($1x10^6$ cells/well) and transfected as described above (Fig. 5). After 24h of incubation, cells were trypsinized for removal from the tissue culture plate and diluted (1: l) in 16% trypan blue (in PBS). Selective permeability of the cell membrane is lost when cells die and these cells passively take up trypan blue and become colored while the live cells exclude the dye. The numbers of live (clear) and dead cells (blue) were counted in a hemocytometer and the average of triplicate analysis presented. The results revealed a decrease in viability with increasing amounts of 2-5A (Figure 5). Transfection of 2-5A in cells pre-treated with IFN β resulted in increased levels of cell death at higher concentrations of trimer. Treatment with 12 μM of trimer in IFN-pre-treated cells resulted in approximately 25% (+/- 5%) cell death while cells treated with only showed approximately 17% (+/- 3.3%). Increased concentrations of 2-5A resulted in higher levels of cell death (50% apoptosis in IFN-pre-treated cells and 35% apoptosis in cells treated with 2-5A alone). The results demonstrate that treatment with 2-5A decreases viability of human ovarian cancer cells, and that this effect is enhanced by treatment with IFN.

Cells were incubated in the presence or absence of 1000 units per ml of IFN-β (Serono) for 16 h prior to transfections with 2-5A or the control dimer. Media containing floating cells and debris was removed to labeled tubes. Cells which remain adhered to the wells were washed with PBS and removed by the addition of trypsin-EDTA (1 ml) (Gibco, BRL) at 37°C for 2 min. The trypsinized cell suspension is combined with the media containing the floating cells and centrifuged at 1,000 g for 5 min at room temperature. The cells are washed once and

resuspended in PBS (1 ml). The cells are stained simultaneously with fluoroscein isothocynate (FITC)-Annexin V [green fluorescence] and propidium iodide (PI) (red fluorescence) for 15 min at room temperature in the dark as described [Pharmingen-Becton Dickinson, San Diego]. Cells are gently resuspended, bivariate flow cytometry using a FACScan is performed and the data analyzed with CellQuest software (Becton Dickinson, San Jose, CA). The results show that apoptosis increased as a function of time. There was 41% and 56% apoptosis after 16 h in the presence of 2-5A alone or 2-5A plus IFN, respectively. Dimer failed to increase apoptosis over background levels in the presence or absence of IFN. Similarly, IFN by itself did not cause apoptosis during the initial 16 h of treatment.

EXAMPLE 4 Treatment of HEY1B cells with a spA2'p5'A2'p5'A2'p3'dC (2-5AdC) and IFN.

To stabilize against enzymatic digestion, we have designed and synthesized a novel analog, SpA2'p5'A2'p5'A2'p3'dC (dC) (Fig. 7). The compound dC is stablized at both termini against enzymes that can degrade. Specifically, the 5'-terminus is protected against phosphatase activity by a thiophosphate group. The 2',3'-terminus is protected against 3' to 5' phosphodiesterase activity linking an inverted deoxycytosine in 2' to 3' linkage to the final (third) 2'-OH of the compound.

The synthesis of spA2'p5'A2'p3'dC (2-5AdC) was carried out on an ABI380B DNA synthesizer. Following the synthesis procedure, the resin was transferred to a vial and treated with ammonium hydroxide-ethanol solution (3:1, v/v) at room temperature for 2h, and heated at 550C for 8h. Solvent was evaporated off in a Speedvac to dryness and 1.5 ml of tetrabutylammonium fluoride (TBAF) (1M) was added prior to overnight incubation at RT. Tetrahydrofuran (TEAA) was added (1ml) to the preparation and the THF was evaporated on the speedvac. dc was purified by RP-HPLC using a RLP-1 column (Hamilton). Effluent was evaporated to dryness and dissolved in deionized water and desalted on Sep-Pak cartridges (Waters) and the final concentration of spA2'p5'A2'p3'dC (dC) was determined to be 3.66 mM.

To determine the ability of 2-5AdC to induce the death of cancer cells, HEY1B ovarian cancer cells were incubated overnight in the absence or presence of IFN-α2a and then transfected with 2-5AdC (Fig. 7). In the absence of IFN treatment, 3 and 6 μM 2-5AdC caused 12.6+6.1% and 44.6+9.1% cell death, respectively. When cells were pretreated with IFN,

however, 3 and 6 μM 2-5AdC caused 20.5+7% and 92.8+0.07% cell death. Therefore, dC has an enhanced cell killing effect on the HEY1B tumor cells.

EXAMPLE 5 SpA2'sp5'A2'sp5'A2'sp5'A

The synthesis of a novel, bioactive and stable analog, pA2'sp5'A2'sp5'A2'sp5', was carried out on a 1 μ mol column on ABI 380B synthesizer. 5'-O-dimethyoxytrityl-N6-benzoyl-3'O-t-butyldimethylsilyladenosine-2'-(2-cyanoethyl-N,5N-diisopropyl)phosphoramidite was purchased from ChemGene (Ashland, MA). 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) solution was from Sigma-Aldrich. Sulfurizing Reagent and other reagents for synthesis were from Glen Research (Sterling, VA). After synthesis, the column was removed from the instrument and the support was transferred into a screw-capped vial. The support was treated with 3 ml ammonium hydroxide-ethanol solution (3/1, v/v) for 2 h at room temperature, then at 55° C for 4 h. The solution was speedvaced down to dryness. The residue was treated with 1 M TBAF-THF solution overnight. THF was removed on the speed vac. 1 ml water was added to the residue, and the resulting mixture vortexed. The crude product was purified by HPLC on a polystyrene reverse phase column using buffer (A: 10 mM tetrabutylammonium dihydrogenphosphate (TBAP), B: 10 mM TBAP in CH_3CN-water, 8/2), and the fractions containing the target compound pooled and dried. Desalting was carried out on Sep-Pak column using 80% methanol as eluant. The tetrabutylammonium salt was transformed into sodium form by ion-exchanging with Dowex 50W (sodium form.)

EXAMPLE 6 In Vivo Treatment of Ovarian Carcinoma with and IFN.

Human ovarian cancer cells (HEY1B) were implanted subcutaneously into the flanks 8 nude (athymic) mice (Fig. 8). When tumors reached volumes of about 100 mm^3, four mice had their tumors directly injected with 50 μl of 5 μM 2-5A complexed with lipofectamine (time Day 0, 3 and 5 in the Figure). Control mice were injected with lipofectamine alone at the same times. On day 6, the treated animals were injected in opposite flanks with 1,000 U of IFN-α2A, continuing with alternate IFN and 2-5A treatments (in the same amounts) on every other day. A dramatic anti-tumor effect was observed with the combination therapy, such that by day 19, average tumor volumes in the lipofectamine control group were 5,454 mm^3, whereas the average tumor volumes in the IFN/2-5A combination therapy group were 3,144 mm^3.

EXAMPLE 7 Treatment of Carcinoma Cells from the Mammary Gland with 2-5A and IFN.

The human adenocarcinoma cell line MCF-7 was obtained from a pleural effusion from the mammary gland of a female patient. The growth of this cell line is inhibited by tumor necrosis factor alpha. An aggressive epithelial carcinoma, MCF-7 cells showed effective killing by 2-5A alone, or with 2-5A and a low concentration of IFN (20U). High concentrations of IFN were effective inhibitors of MCF-7 cell growth , even in the absence of 2-5A. Low concentration of 2-5A decreased viability by 50% (4 μM in the presence or absence of 20 U/ml IFN). These results demonstrate that the MCF-7 cells were sensitive to the antiproliferative/apoptotic effects of 2-5A and IFN.

EXAMPLE 8 Treatment of Bladder Carcinoma Cells with 2-5A and IFN.

Bladder epithelial cells RT4 and T24 cells were transfected with 2-5A (0-36μM) in the presence or absence IFN over a period of 48h. T24 cells are transitional bladder carcinoma cells which responded to 2-5A treatment at higher IC-50 concentrations of 2-5A (32 μM). The cell viability decreased further with IFN pre-treatment and the IC-50 concentrations of 2-5A were decreased to 30, 20 and 18 μM for 20, 100, and 1,000 U/ml IFN, respectively. RT4 cells are transitional bladder epithelial papillomas, a much less invasive form of cancer. These cells resisted treatment to both and IFN. The IC-50 concentrations remained greater than 36 μM except for 1,000 U/ml IFN, where the IC-50 concentration of 2-5A decreased to 12 μM. The enhanced anti-cellular effect of 2-5A and IFN in the more aggressive T24 transitional carcinoma cells as compared with RT4 papilloma cells suggests a preferred therapeutic window for treating bladder cancer.

EXAMPLE 9 Treatment of Fibrosarcoma Cells with 2-5A and IFN.

Fibroblasts HT 1080 and WI-38 cells were transfected with (0-2μM) in the presence or absence of IFN in order to compare the ability of RNase L to decrease cell viability through apoptosis. The HT1080 fibrosarcoma cells were very sensitive to the effects of 2-5A activation of RNaseL and apoptosis induction. HT 1080 fibrosarcoma cells had an IC-50 concentration of only 0.25 μM after 72h of treatment of 2-5A alone and was decreased by the addition of IFN pretreatment. In contrast, the normal lung fibroblast primary cells WI-38, required almost 1 μM

of 2-5A (72h) in the absence of IFN.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims

CLAIMS

What is claimed is:

1. A method of treating malignancies in a subject comprising administering a therapeutically effective amount of 2-5A or a biostable analog thereof to the subject.

2. The method of claim 1 further comprising the step of administering a therapeutically effective amount of interferon (IFN) to the subject, wherein the IFN and the 2-5A or the biostable analog thereof are administered concurrently or sequentially.

3. The method of claim 2 wherein the IFN and the 2-5A or the biostable analog thereof are administered at a concentration and for a time sufficient to stop or slow the growth of the malignancy.

4. The method of claim 1 wherein the IFN and 2-5A or the biostable analog thereof are in different pharmaceutical compositions.

5. The method of claim 2 wherein the IFN and 2-5A or the biostable analog thereof are contained within the same pharmaceutical composition.

6. The method of claim 1 wherein the subject has or is suspected of having ovarian carcinoma, glioblastoma, breast carcinoma, or fibrosarcoma.

7. The method of claim 2 wherein the subject has or is suspected of having a malignancy selected from the group consisting of hairy cell leukemia, Kaposi's sarcoma, CML, B-cell lymphoma, T-cell lymphoma, melanoma, myeloma, renal cell carcinoma, ovarian carcinoma, breast carcinoma, bronchogenic carcinoma, bladder carcinoma, gastrointestinal carcinoma and acute leukemia.

8. The method of claim 1 wherein the 2-5A or the biostable analog thereof is complexed with a cationic lipid carrier or a liposome.

9. The method of claim 8 wherein the complex further comprises a molecule which targets the complex to cancer cells.

10. The method of claim 1 wherein the 2-5A or the biostable analog thereof is administered by direct or oral administration.

11. The method of claim 1 wherein the 2-5A of the biostable analog thereof is administered by intravenous infusion.

12. The method of claim 2 wherein the IFN is a type I IFN or a consensus IFN.

13. A method of treating a subject having or suspected of having a viral disease, comprising :

(a) administering IFN to the subject, and

(b) administering 2-5A or a biostable analog thereof to the subject, wherein the IFN and 2-5A or the biostable analog thereof are administered concurrently or sequentially.

14. The method of claim 13 wherein the IFN and 2-5A or the biostable analog thereof are administered at a concentration and for a time sufficient to ameliorate the pathological effects of the viral disease.

15. The method of claim 13 wherein the IFN and 2-5A or the biostable analog thereof are in different pharmaceutical compositions.

16. The method of claim 13 wherein the IFN and 2-5A or the biostable analog thereof are contained within the same pharmaceutical composition.

17. The method of claim 13 wherein the subject has or is suspected of having hepatitis C, hepatitis B, or a viral infection caused by a picornavirus.

18. A method of inducing apoptosis in a cancer cell comprising:

(a) administering IFN to the cell, and

(b) administering 2-5A or a biostable analog thereof to the cell.

19. The method of claim 18 wherein in the 2-5A or the biostable analog thereof is complexed with a cationic lipid carrier or a liposome.

20. The method of claim 18 wherein the 2-5A or analog thereof is selected from the group consisting of the trimeric species, the tetrameric species, the pentameric species, and combinations thereof.

21. A pharmaceutical composition comprising 2-5A or a biostable analog thereof and a pharmaceutically acceptable carrier.

22. The pharmaceutical composition of claim 21 wherein the biostable analog is SpA2'sp5'A2'sp5'A2'sp5'A, or SpA2'p5'A2'p3'dC, or SpA2'p5'A2'p5'A2'p5'A-2'-OMe.

23. The pharmaceutical composition of claim 21, wherein the composition further comprises IFN.

Figure 1

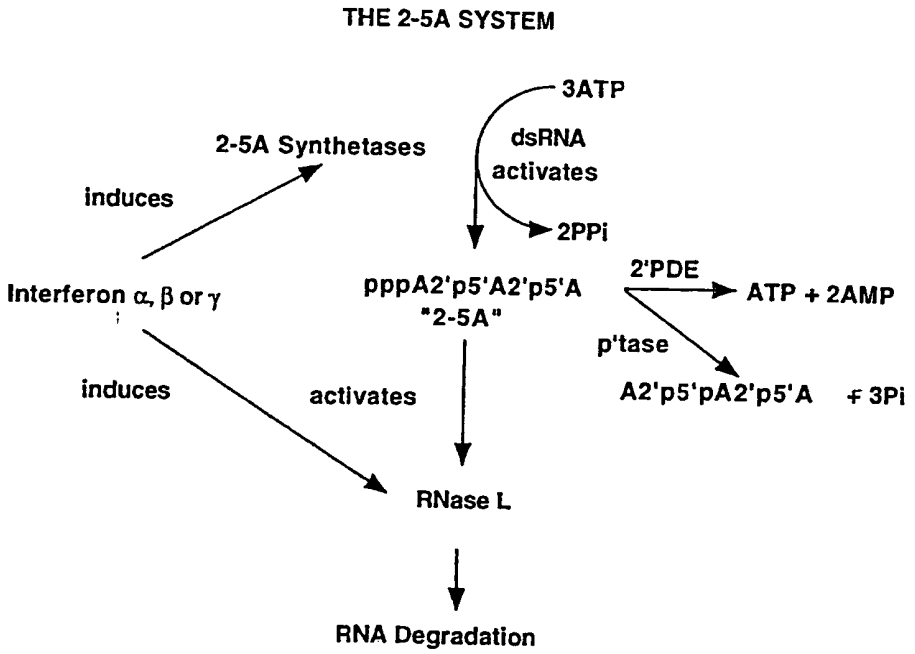
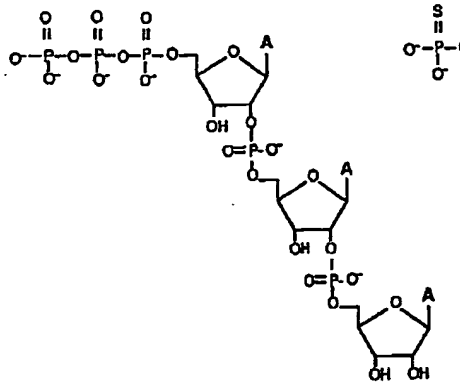


Fig 1

A. Trimer 2-5A



B. Trimer 2-5AdC

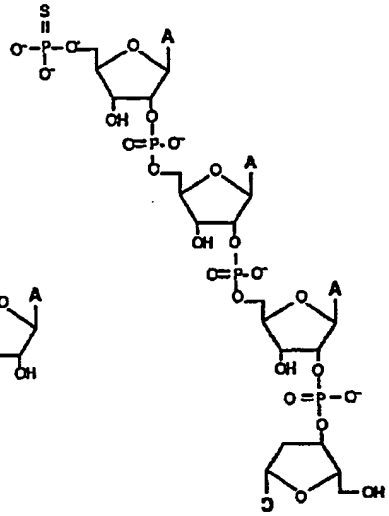


Fig 1

Figure 3

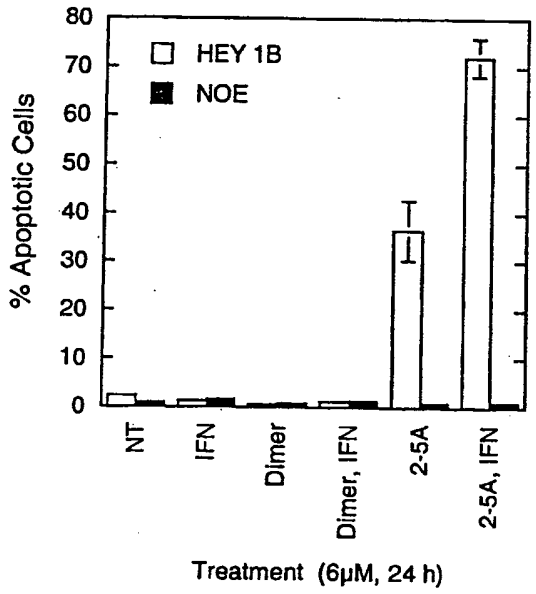
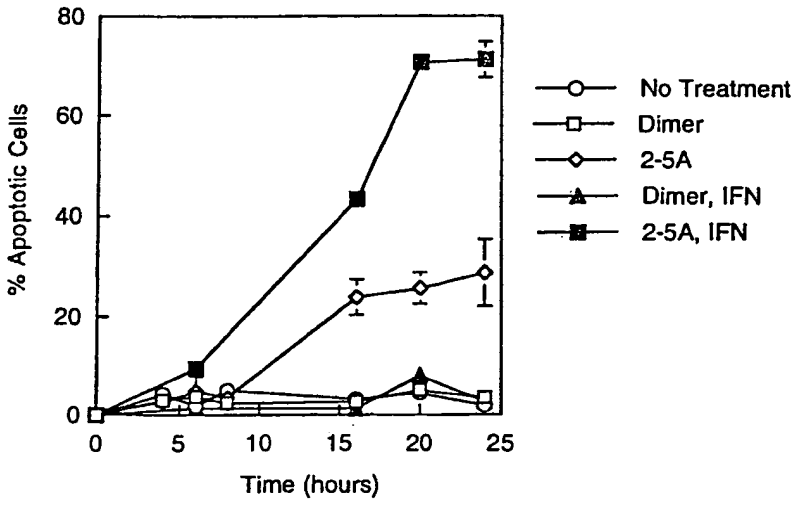

80
70
60
50
40
30
20
10
0
% Apoptotic Cells
HEY 1B
NOE
NT
IFN
Dimer
Dimer, IFN
2-5A
2-5A, IFN
Treatment (6μM, 24 h)

Figure 4


80
60
40
20
0
% Apoptotic Cells
0
5
10
15
20
25
Time (hours)
No Treatment
Dimer
2-5A
Dimer, IFN
2-5A, IFN

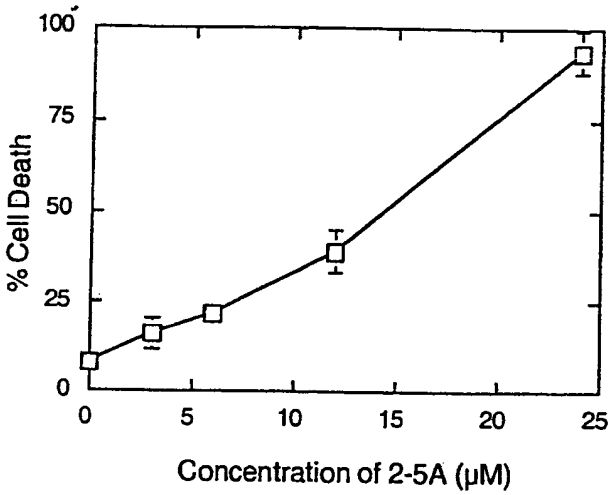
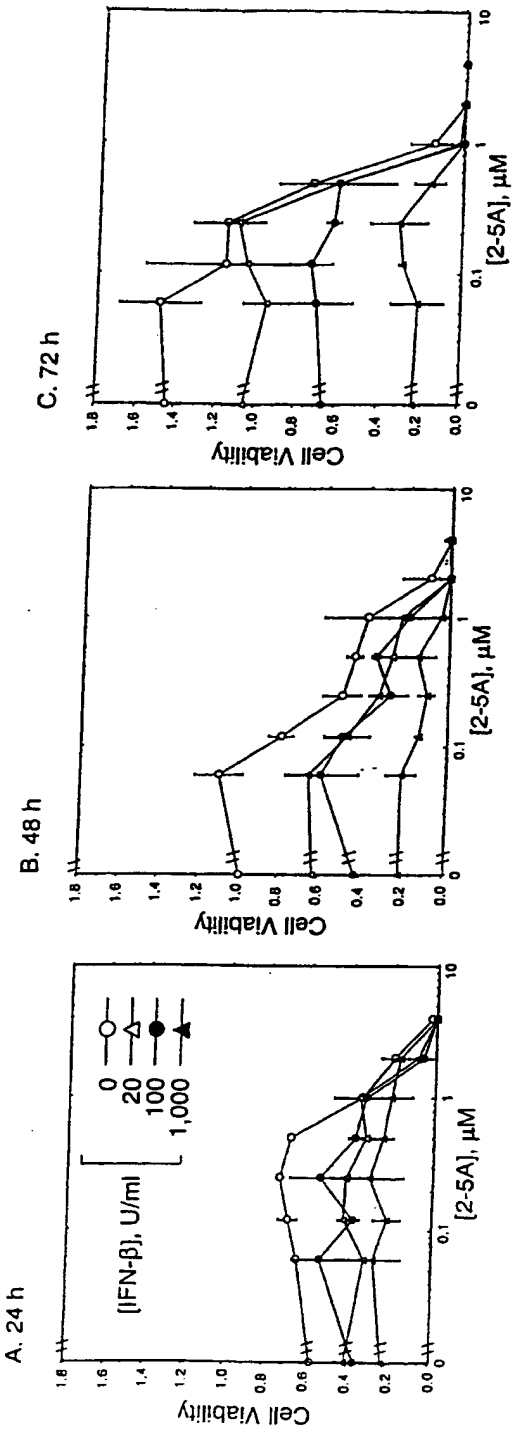


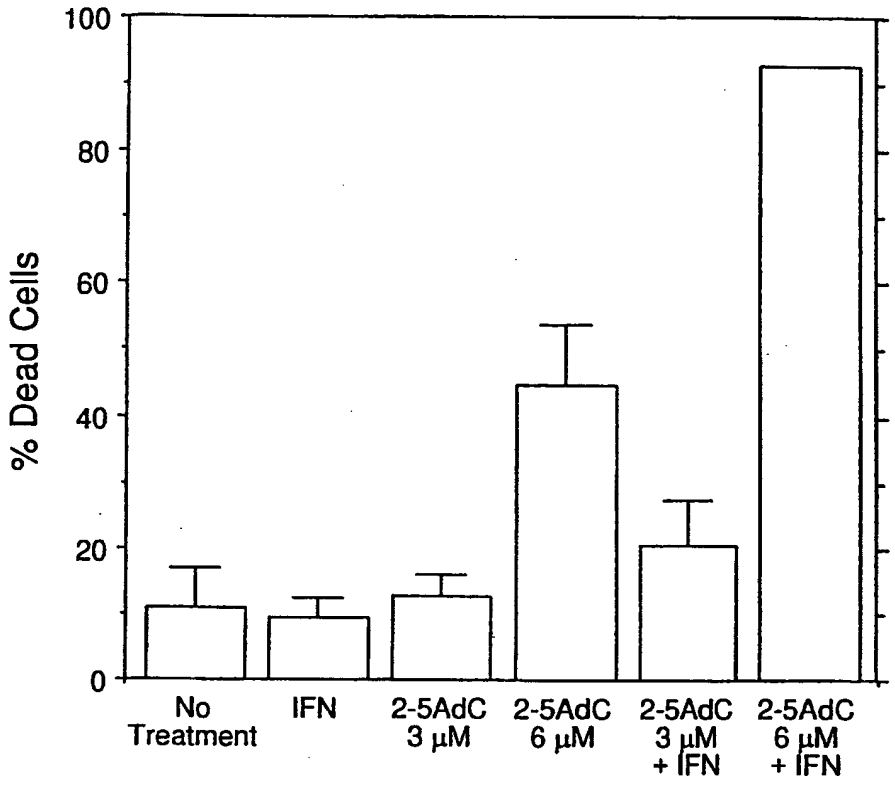
Fig 5



317

Fig. 6

Figure 7


100
80
60
40
20
0
% Dead Cells
No Treatment
IFN
2-5AdC 3 μM
2-5AdC 6 μM
2-5AdC 3 μM + IFN
2-5AdC 6 μM + IFN

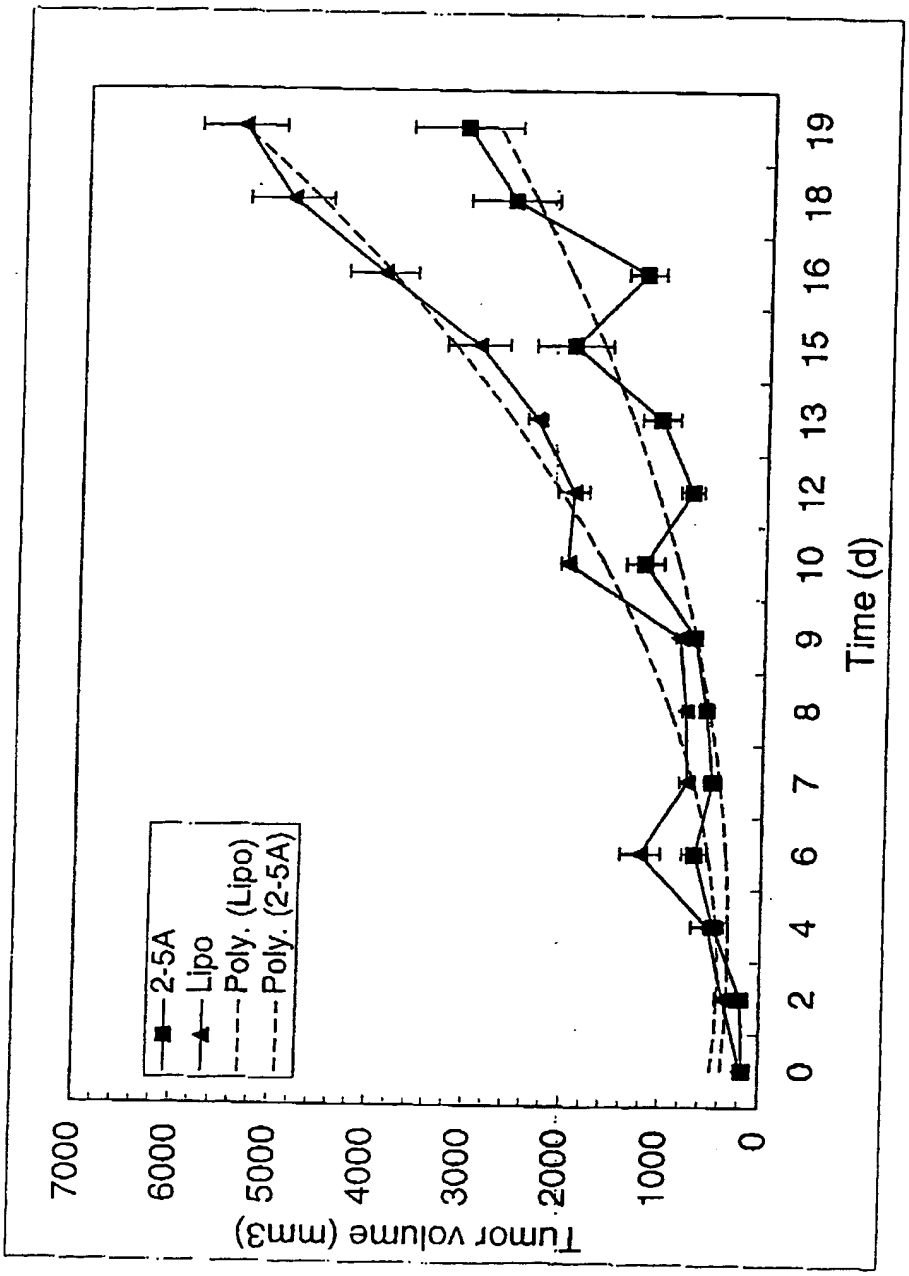

Tumor volume (mm3)
7000
6000
5000
4000
3000
2000
1000
0
0 2 4 6 7 8 9 10 12 13 15 16 18 19
Time (d)
2-5A
Lipo
Poly. (Lipo)
Poly. (2-5A)

Fig. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/41038

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 31/70
US CL :514/44, 46; 424/85.7, 85.4
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/44, 46; 424/85.7, 85.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST
oligoadenylate, carcinoma, fibrosarcoma, glioblastoma, hepatitis, picornavirus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,908,621 A (GLUE et al.) 01 June 1999, abstract, col. 5 line 1 to col. 6, line 52.	13-17, 21-23
Y	US 5,550,111 A (SUHADOLNIK et al.) 27 August 1996, abstract, claims 29-31.	13-17, 21-23
Y, P	US 5,962,431 A (BUDOWSKY et al.) 05 October 1999, see entire document.	13-17, 21-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
18 DECEMBER 2000

Date of mailing of the international search report
25 JAN 2001

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